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Feminization of Male Brown Treesnake Methyl Ketone Expression via Steroid Hormone Manipulation

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Abstract

Pheromones are useful tools for the management of invasive invertebrates, but have proven less successful in field applications for invasive vertebrates. The brown treesnake, *Boiga irregularis*, is an invasive predator that has fundamentally altered the ecology of Guam. The development of control tools to manage *Boiga* remains ongoing. Skin-based, lipophilic pheromone components facilitate mating in brown treesnakes, with females producing the same long-chain, saturated and monounsaturated (ketomonoene) methyl ketones known to function as pheromones in garter snakes, *Thamnophis sirtalis*. *Boiga* also express novel, diunsaturated methyl ketones (ketodienes) with a purported function as a sex pheromone. In our study, we implanted 17 β-estradiol in adult male brown treesnakes in order to manipulate methyl ketone expression as sex attractants, an effect that would mirror findings with garter snakes. Specifically, estrogen promoted production of two ketomonoenes, pentatriaconten-2-one and hexatriaconten-2-one, and suppressed production of one ketodiene, heptatriacontadien-2-one. In bioassays, estrogen-implanted males elicited tongue-flicking and chin rubbing behavior from unmanipulated males, though the responses were weaker than those elicited by females. On Guam, wild males exhibited greatest responses to whole female skin lipid extracts and only weak responses to the methyl ketone fractions from females and implanted males. Our results suggest that sex identity in brown treesnakes may be conferred by the ratio of ketomonoenes (female) to ketodienes (male) from skin lipids and may be augmented by a sex-specific endocrine signal (estradiol). However, a blend of long-chain methyl ketones alone is not sufficient to elicit maximal reproductive behaviors in male *Boiga*.

Keywords Pheromone · Estrogen · Snake · *Boiga* · Reproduction · Invasive species

Introduction

Invasive vertebrates, especially predatory species, pose significant problems to the implementation of management strategies to rescue threatened, endemic taxa and sensitive ecosystems. A hallmark invasive predator is the brown treesnake, *Boiga irregularis*. Since its introduction in the 1940s to the island of Guam, the brown treesnake has extirpated the majority of the island's native bird species and many native small mammal and lizard species (Rodda and Fritts 1992; Savidge 1987; Wiles et al. 2003). Through these faunal impacts, the brown treesnake is attributed with altering the native ecology of Guam (Mortensen et al. 2008). For example, the loss of endemic bird species disrupted crucial mutualisms and caused a subsequent decline of rare plants (Rogers et al. 2017; Traveset and Richardson 2006). To ameliorate the impacts of *Boiga* on Guam, primary management strategies have centered on trapping to maintain snake-free areas and/or distributing lethal baits specifically designed for wide-area suppression (Kimball et al. 2016; Smith et al. 2016). The use of conspecific signals, especially chemicals, has been less explored, though there is considerable evidence that *Boiga* uses chemical communication to find and choose mates (Greene and Mason 1998; Mathies et al. 2013).

Pheromones can be powerful control tools to manage invasive species. For example, pheromone-based technologies are broadly employed to trap (and kill) pest insects (El-Sayed et al. 2006; Hwang and Lindroth 1997). Synthetic sex pheromones can be highly effective, such as their use in the

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eradication of European gypsy moths, *Lymantria dispar*, via mating disruption (Gaston et al. 1967; McNeil 1991; Reardon et al. 1998). Similarly, pheromone-based traps greatly improve management of invasive species, such as the European corn borer, *Ostrinia nubilalis*, which causes significant economic losses in agriculture on multiple continents (Albajes et al. 2002; Bohnenblust et al. 2014). In pest vertebrate species, synthetic sex pheromones have been successfully implemented in traps for multiple species of rodents [e.g., brown rats, *Rattus norvegicus* (Takács et al. 2016); house mice, *Mus musculus* (Musso et al. 2017; Takács et al. 2017)]. Though still in the early stages of development, pheromone mixtures show promise for enhancing trap-based management approaches for sea lampreys, *Petromyzon marinus*, in the Great Lakes region of the U.S. (e.g., mixtures of pheromone components, Johnson et al. 2015). For invasive amphibians, such as cane toads, *Rhinella marina*, in Australia, conspecific chemical cues have been strongly implicated as useful control agents (Clarke et al. 2016; Saunders et al. 2010). As for other vertebrate, and countless invertebrate, pests, it is thus of interest to know if chemical cues have utility for managing invasive reptiles such as the brown treesnake.

All snake species studied to date show the same pattern of reproductive chemical ecology, in which males exhibit behaviors in response to female chemical signals from the integument (Mason 1992; Parker and Mason 2011). The same may be said for most, if not all, lizard species (Mason and Parker 2010). Brown treesnakes follow this pattern in that males use skin-based pheromone components from females to coordinate reproduction (Greene and Mason 1998). Male brown treesnakes follow female chemical trails and exhibit reproductive behaviors (tongue-flicking, chin rubbing) toward isolated lipid extracts in bioassays (Greene and Mason 1998, 2000). In their skin lipids, female treesnakes produce a series of long-chain (C_{33}-C_{37}) saturated, monounsaturated (ketomonoene), and diunsaturated (ketodiene) methyl ketones, with the ketodienes comprising the majority of the ketones of this species (Murata et al. 1991). However, male *Boiga* have not been examined previously. Another snake species, the red-sided garter snake, *Thamnophis sirtalis parietalis*, produces the same blend of saturated and monounsaturated methyl ketones, with the ketomonoenes, especially the longest ones, conferring attractiveness to females (LeMaster and Mason 2002; Mason et al. 1989). The sexual attraction pheromone of garter snakes can be manipulated using steroid hormone implants, as shown by female pheromone expression in estradiol-treated males (Parker and Mason 2012). Removal of the implants returned males to an unattractive state, indicating the estrogen effect was reversible (activational) rather than permanent (organizational) (Parker and Mason 2012). Specifically, the ketomonoenes were upregulated by estrogen but inhibited by testosterone, while testosterone promoted saturated methyl ketone production (Parker and Mason 2014). Therefore, in garter snakes, at least, sex hormones activate specific, sexually dimorphic patterns of chemical signal expression.

The purposes of this study were to determine if methyl ketone expression in brown treesnakes was 1) sexually dimorphic, 2) responsive to hormonal (estradiol) manipulation, and 3) detectable by males. If hormone manipulation can activate expression of biologically useful pheromone components in brown treesnakes, there is potential to use these compounds in management efforts on Guam. Specifically, the methyl ketones of brown treesnakes and garter snakes are cost prohibitive to synthesize, especially in light of the quantities needed for field applications. However, if hormone manipulation of live snakes yielded appreciable quantities of sexually attractive blends of pheromone components, such biosynthesis could be a feasible avenue for future research.

Methods and Materials

Captive Brown Treesnake Housing and Manipulation Wild-caught brown treesnakes were imported from Guam and maintained in captivity at the U.S. Department of Agriculture's National Wildlife Research Center (NWRC) in Ft. Collins, CO, in clear plastic containers lined with absorptive bedding and containing a hidebox. Water was provided ad libitum, and snakes were fed a diet of dead rodents once a week. Females were maintained at 19 °C for 60 d to induce a vitellogenic state and increase attractiveness, following the work of Mathies (Mathies and Miller 2003; Mathies et al. 2013), before returning them to their typical holding temperature (approx. 24 °C). Bioassays were conducted after this (see below).

To manipulate hormone levels, previously validated methods were used to deliver intraperitoneal steroid hormone implants (Parker and Mason 2012, 2014). Seven males ($N = 7$) received a silastic tube (0.033 cm ID × 0.05 cm OD × 2 cm long) packed with crystalline 17 β-estradiol (Sigma-Aldrich, St. Louis, MO, USA) and sealed with silicone (Dow Corning, Auburn, MI, USA). Snakes were anaesthetized with isoflurane before making a small lateral incision (5 mm) between the first and second dorsal scale rows, adjacent to the location of the anterior testis, and the implant was delivered into the fat bodies. The incision was then sutured closed and the snakes monitored until the suture was shed with the snake's next ecdysis. The estradiol implants were toxic to the brown treesnakes, and four of the males died 2–3 months following implantation, reducing the number of animals that could be used in bioassays at the NWRC.

Chemical Analyses Shed skins were collected from implanted and unmanipulated males ($N = 6$) and females ($N = 5$) over the course of the year and kept dry until lipids extracted from the skins (Greene and Mason 1998). Whole shed skins were

immersed in hexane (FisherScientific, Waltham, MA, USA) for 24 hr, followed by complete rotary evaporation, yielding total skin lipid mass (mg). Extracts were then fractionated using established methods (Mason et al. 1989; Parker and Mason 2012). Briefly, hexane extracts were fractionated on alumina columns (activity III; Sorbtech Technologies, Norcross, GA, USA) with diethyl ether (Sigma-Aldrich, St. Louis, MO, USA) as the mobile phase. Fractions were pooled in the following order: fractions 1–3 (0% ether), 4–6 (2%), 7–9 (4%), 10–12 (8%). These pooled fractions were named F1 (fractions 1–3), F2 (fractions 4–6), F3 (fractions 7–9), and F4 (fractions 10–12). The methyl ketones eluted primarily in F3, though trace amounts were also found in F4. For bioassays on Guam (see below), F1 and F2 (F1 + 2; no methyl ketones) and F3 and F4 were combined (F3 + 4; methyl ketones) to yield less and more polar, respectively, samples whose behavioral properties could be tested. After they were weighed, pooled fractions were reconstituted in hexane at 1 mg/ml.

Pooled fractions (e.g., F1) were analyzed for an individual with an Agilent 6890 gas chromatograph (GC) equipped with an Agilent 5973 mass selective detector (MS) and HP 7683B autosampler (Agilent Inc., Santa Clara, CA, USA). Aliquots (2 μl) of the 1 mg/ml samples were injected onto a ZB-1MS (30 m × 0.32 mm I.D., 0.25 μm film thickness) capillary column (Phenomenex, Torrance, CA, USA), with helium as carrier gas (2.3 ml.min^{-1}). All injections were made in splitless mode (30 sec). The inlet temperature was 280 °C, the MS interface temperature 250 °C, and the ion source temperature 230 °C. The column oven temperature was held initially at 70 °C for 1 min, increased to 210 °C at 30 °C.min^{-1}, held at 210 °C for 1 min, increased to 335 °C at 5 °C.min^{-1} and held for 5 min. The mass spectrometer was operated in positive electron impact ionization (EI) mode at 69.9 eV ionization energy and a scan range of *m/z* 33–800. Chromatograms and mass spectra were evaluated using Agilent Chemstation software (version B.04.03; Agilent Inc., Santa Clara, CA, USA). Individual compounds were identified by comparing mass spectral data and retention times to those of published data (Mason et al. 1989, 1990; Murata et al. 1991). Peak integration data were obtained using Chemstation and then converted to proportions of total methyl ketone composition to control for differences in sample concentration prior to statistical analysis.

Bioassays Two sets of bioassays were performed. The first were conducted with captive brown treesnakes at the NWRC facilities in Fort Collins, CO. All snakes were subjected to a 60 d cooling period at 19 °C, as described above, prior to testing approximately one week after returning to normal holding temperature (ca. 24 °C). First, males ($N = 4$ control males; $N = 3$ implanted males) were tested for behavioral discrimination of chemical cues in their home cages. Female skin lipid extract from a neat, pooled shed skin sample ($N = 3$ female shed skins; 5 mg of lipid/ml of hexane) was pipetted onto one half of a Whatman filter paper (9 cm wide, grade 1) and the hexane allowed to evaporate. A peanut oil extract (5 mg/ml of hexane) was used as a control and placed on the other half of the filter paper. Total tongue-flicks and total time (sec) per section of the filter paper were quantified to determine rate of tongue-flicking (RTF; tongue-flicks/min). The RTF is a direct measurement of chemosensory sampling rate in squamate reptiles that can be used to test for chemical discrimination by focal animals.

For the second bioassay at NWRC, individuals ($N = 3$ females; $N = 4$ control males; $N = 3$ implanted males) were paired in a factorial design for 30 min observation trials, and their behaviors recorded. Snake pairs were placed into a rectangular observation chamber (1 × 1 × 2.5 m) with a Plexiglas door and the floor covered with disposable paper that was changed between every trial to minimize odor contamination. We quantified RTF when the snakes were in contact with one another and also recorded chin rubbing behavior from males (chin rubs/min). Chin rubbing is an unequivocal mating behavior that reproductive male snakes display toward females during courtship (Greene and Mason 2000; Parker and Mason 2011). An implanted male was paired singly with one control male per bioassay ($N = 4$ trials per implanted male, $N = 12$ trials total). After all control males had been tested with each implanted male, control male responses were averaged per implanted male. The same was done with control male responses when paired with females ($N = 12$ trials total). Therefore, responses reflected the average control male response to a female or an implanted male.

Following the bioassays at NWRC, we next ran bioassays with wild brown treesnakes at the Guam National Wildlife Refuge in collaboration with the U.S. Geological Survey. Adult male ($N = 20$) and female ($N = 20$) brown treesnakes were captured on Guam and temporarily housed, individually, in ventilated 20 l buckets fitted with transparent lids. The room was maintained on a 12 hr: 12 hr L:D cycle at 28 °C. Water was provided ad libitum and snakes were fed frozen mice once per week for a two week acclimation period. Mating trials were conducted in behavioral arenas as follows. Pop-up tents ($N = 2$; 1 m × 1 m × 1.4 m; Sport Pods, Under the Weather, Cincinnati, OH, USA) were cleaned with 70% ethanol and lined with white butcher paper. The arena exterior was covered with opaque paper to minimize conflicting visual stimuli, and a single red bulb was suspended from the top of the arena for illumination. All bioassays were conducted between 2000 and 0200 hr, corresponding to the typical active period for this species on Guam.

Males were first prescreened for positive reproductive behavior by assaying male-female pairs in arenas. A single female was placed in an arena and given 20 min to acclimate before introducing a single male. Reproductive behaviors were noted, and males were determined to be reproductive if

they displayed two behaviors: tongue-flicking directed at the female's skin and chin rubbing on the female's dorsum (Greene and Mason 2000). This assay also enabled assessment of female attractiveness and validated that larger females in better condition [ratio ln(mass)/ln(length)] elicited the greatest response from males.

Male pairs were tested in three different trials: female lipid extract vs. male lipid extract (F vs M), female F1 + 2 (0% and 2% polarity fractions) vs female F3 + 4 (4% and 8% polarity fractions; methyl ketone fractions), and implanted male F1 + 2 vs implanted male F3 + 4. A previous study reported that male brown treesnakes showed the strongest responses to the neutral lipid fractions compared to the more polar lipids (Greene and Mason 1998); thus, both F1 + 2 and F3 + 4 were tested. To test the whole skin lipid extracts and the fractionated samples, courting males that had been prescreened ($N = 10$) were size-matched and placed as a pair ($N = 2$) into a testing arena. Single males were tested initially, but they never spent time exploring the arena even after prolonged acclimation periods of 1 hr; instead, they would first attempt to escape the arena and then eventually sit in one of the corners in a defensive strike coil. Further, previous work showed that chemosensory behaviors of male brown treesnakes were strongest in a captive setting only after staging male-male combat (Greene and Mason 1998). Therefore, we paired males for our tests of chemical isolates. After the male pairs acclimated for 20 min, the samples were pipetted onto damp Whatman filter papers (18.5 cm diam., grade 1), placed into opposite sides of the arena. Filter papers were dampened with deionized water. Total tongue-flicks and total time (sec) per filter paper were quantified to determine RTF (tongue-flicks/min). Trials proceeded until both males had at least sampled each filter paper twice or 20 min had elapsed. The arena paper was removed, the arena rinsed with 70% ethanol, and the paper replaced before proceeding to the next male trial pairing. Males were tested only once per night. Male-male behaviors were also recorded qualitatively to confirm that males were in breeding condition and would exhibit stereotypical combat behaviors (Greene and Mason 2000). Any reproductive behaviors typical of male-female interactions (e.g., chin rubbing, head jerking) were recorded but were too infrequent to quantify with the tests of chemical isolates.

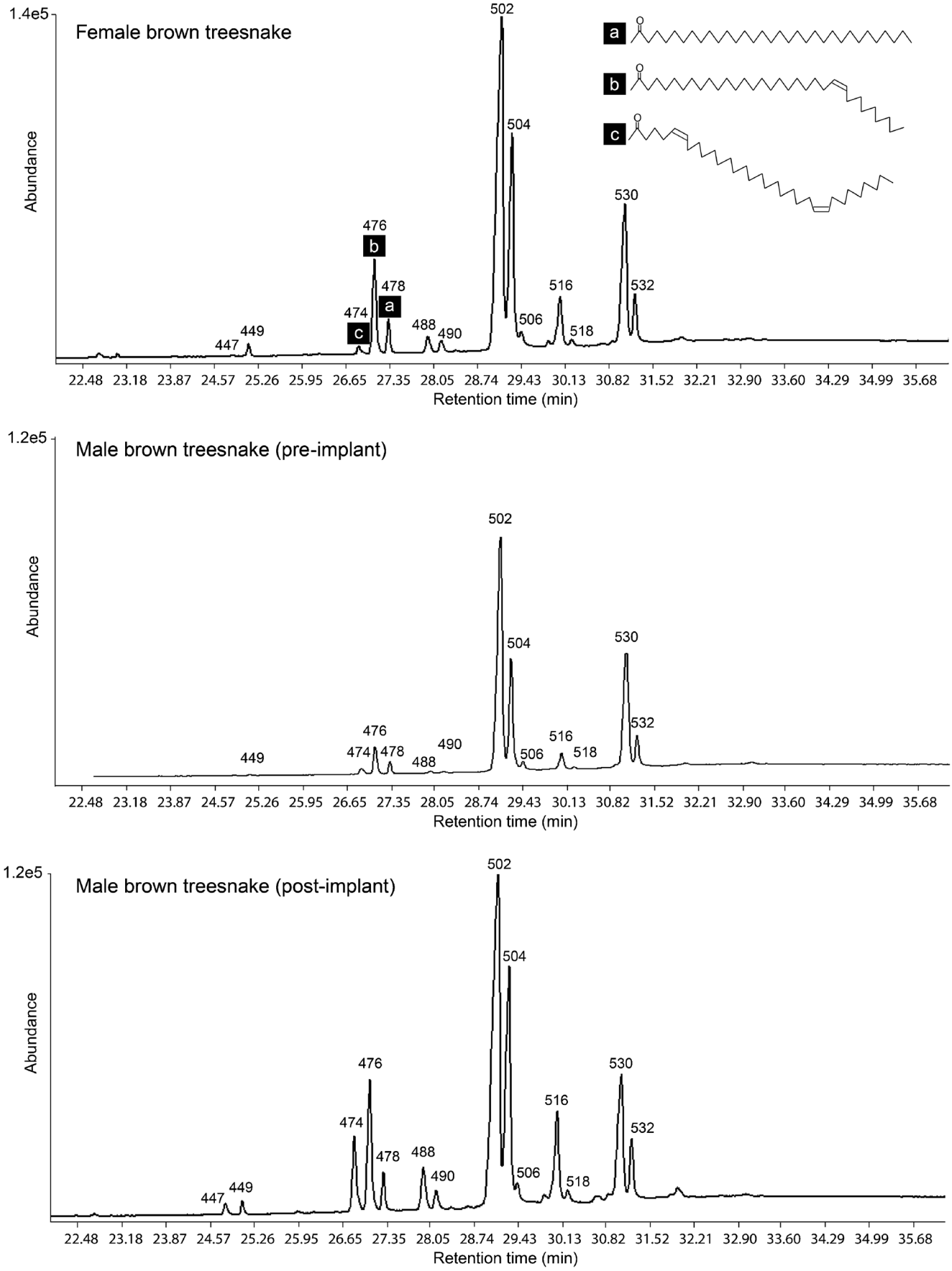
Statistical Analyses Differences in methyl ketone composition between the sexes were evaluated by two-way (methyl ketone, sex) repeated-measures ANOVA followed by adjusted pairwise comparisons (Bonferroni). The effects of hormone implantation on lipid composition were tested with a two-way (methyl ketone, implant status) repeated-measures ANOVA (pre- vs. post-implant) followed by pairwise comparisons (simultaneous Bonferroni tests). Following identification of sexually dimorphic methyl ketone expression, the ratio of the 504 Da ketomonoene to the 530 Da ketodiene was compared across groups using either a Student's t-test (female vs pre-implant male) or a paired t-test (pre- vs post-implant male). The RTF data from the filter paper tests at Ft. Collins were tested with paired t-tests. For the behavioral data from the control males interacting with females vs. implanted males, a one-way (sex) repeated-measures ANOVA was used to determine if control males exhibited different tongue-flicking and chin rubbing behaviors to focal animals. For the bioassays with wild males on Guam, a one-way (filter paper cue) repeated-measures ANOVA, followed by pairwise comparisons (Student-Neuman-Keuls), was used to determine if males chemically discriminated among the types of lipids presented. For all statistical tests, α was set at 0.05. Marginally significant differences ($0.1 > P > 0.05$) were reported as well.

Results

Both sexes produced detectable amounts of all the methyl ketones identified by Murata et al. (1991) and Mason et al. (1989) (Fig. 1). Analysis of the methyl ketone extracts revealed that female and male brown treesnakes varied in the proportion of specific compounds within the blend of ketones present (interaction: $F_{15,175} = 3.02$, $P < 0.001$; Fig. 2). Females produced a greater proportion of only a single ketomonoene [pentatriaconten-2-one (504 Da)] than did pre-implant males ($t_9 = 5.27$, $P < 0.001$). Males produced a greater proportion of a single ketodiene [heptatriacontadien-2-one (530 Da)] than did females ($t_9 = 4.07$, $P < 0.001$). All other comparisons for specific methyl ketones between females and pre-implant males were not significant ($P > 0.10$). When comparing female ketone composition to that of post-implant males, there was not a significant interaction ($F_{15,175} = 0.59$, $P = 0.87$), but there was an effect of snake type (female vs post-implant male) on methyl ketone proportion ($F_{1,175} = 99.68$, $P < 0.001$). Females expressed a higher proportion of pentatriaconten-2-one than post-implant males ($t_9 = 2.45$, $P = 0.015$). All other comparisons for specific methyl ketones between females and post-implant males were not significant ($P > 0.10$).

Male methyl ketone expression patterns were altered following estrogen implantation (interaction: $F_{15,191} = 2.48$, $P = 0.005$). The interaction between implant status and methyl ketone identity was driven by three compounds. Pentatriaconten-2-one and hexatriaconten-2-one (518 Da) increased in proportion in post-implant males compared to pre-

Fig. 1 Representative gas chromatograms for female (top), control male (middle) and estradiol-implanted male (bottom) brown treesnake methyl ketone fractions. Brown treesnakes produce three types of methyl ketones: (**a**) saturated, (**b**) mono-unsaturated (ketomonoenes), (**c**) di-unsaturated (ketodienes). Numbers above peaks are molecular ion *m/z*. Compound names are given in Fig. 2


1.4e5
Female brown treesnake
Abundance
a
b
c
502
504
530
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478
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532
474
506
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518
449
447
22.48 23.18 23.87 24.57 25.26 25.95 26.65 27.35 28.05 28.74 29.43 30.13 30.82 31.52 32.21 32.90 33.60 34.29 34.99 35.68
Retention time (min)
1.2e5
Male brown treesnake (pre-implant)
Abundance
502
504
530
476
532
516
490
474
478
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518
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22.48 23.18 23.87 24.57 25.26 25.95 26.65 27.35 28.05 28.74 29.43 30.13 30.82 31.52 32.21 32.90 33.60 34.29 34.99 35.68
Retention time (min)
1.2e5
Male brown treesnake (post-implant)
Abundance
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22.48 23.18 23.87 24.57 25.26 25.95 26.65 27.35 28.05 28.74 29.43 30.13 30.82 31.52 32.21 32.90 33.60 34.29 34.99 35.68
Retention time (min)

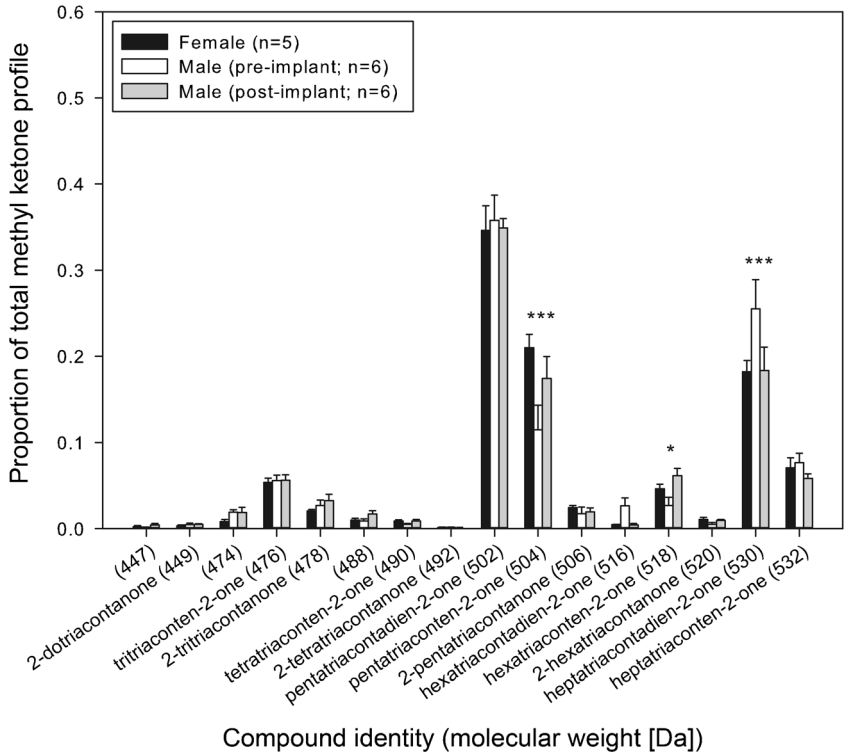

Fig. 2 The proportions of methyl ketones in skin lipid profiles of male brown treesnakes was altered by estradiol implantation. Estradiol in males promoted the production of pentatriaconten-2-one and hexatriaconten-2-one (both ketomonoenes) and suppressed production of heptatriacontadien-2-one (a ketodiene). Female values are depicted for comparison. Bars represent means (+SEM). Asterisks indicate differences between pre- and post-implant males (***$P < 0.001$, *$P < 0.05$). Unnamed compounds were not identified but presumed to be ketodienes based on spectra for these compounds compared to the larger, previously identified ketodienes by Murata et al. (1991)

implant ones ($t_5 = 3.57$, $P < 0.001$; $t_5 = 2.08$, $P = 0.04$, respectively). As with the comparison to females, heptatriacontadien-2-one was produced in greater quantities in males pre-implant compared to post-implant ($t_5 = 4.33$, $P < 0.001$). All other comparisons for specific methyl ketones between pre- and post-implant males were not different ($P > 0.10$).

Because the expression patterns of only two methyl ketones were sexually dimorphic, the ratio of these two ketones, pentatriaconten-2-one (female-biased) and heptatriacontadien-2-one (male-biased), was calculated (hereafter, referred to as the 504:530 ratio). The 504:530 ratio for females (1.18 ± 0.11; mean ± SEM) was higher than that in pre-implant males (0.53 ± 0.15; $t_9 = 3.20$, $P = 0.005$) (Fig. 3). The 504:530 ratio was also higher for post-implant (1.11 ± 0.22) compared to pre-implant ($t_5 = 2.15$, $P = 0.041$) males. Females and post-implant males did not differ in their 504:530 ratios ($t_9 = 0.25$, $P = 0.80$).

All male brown treesnakes at the NWRC in Ft. Collins showed chemosensory discrimination between female lipids and the control lipid sample (46.66 ± 6.38 tongue-flicks/min vs. 19.66 ± 2.29, respectively; $t_5 = 5.26$, $P = 0.001$). In the behavioral pairings, control male mean RTFs toward females (72.75 ± 5.48 tongue-flicks/min) did not differ from those directed to estrogen-implanted males (73.58 ± 7.02; $F_{1,7} = 0.028$, $P = 0.87$) (Fig. 4). Rates of chin rubbing were higher when control males were paired with females (33.83 ± 4.61 chin rubs/min) compared to implanted males (2.25 ± 1.93; $F_{1,7} = 115.25$, $P = 0.002$). Qualitatively, in the male-female behavioral trials, females exhibited overt solicitation behaviors (e.g., body bridging, rubbing, tongue-flicking) once they tongue-flicked males; these behaviors are hypothesized to have accelerated male sexual behaviors (e.g., chin rubbing). All estrogen-implanted males exhibited stereotypical courtship behavior toward females, indicating that the implants

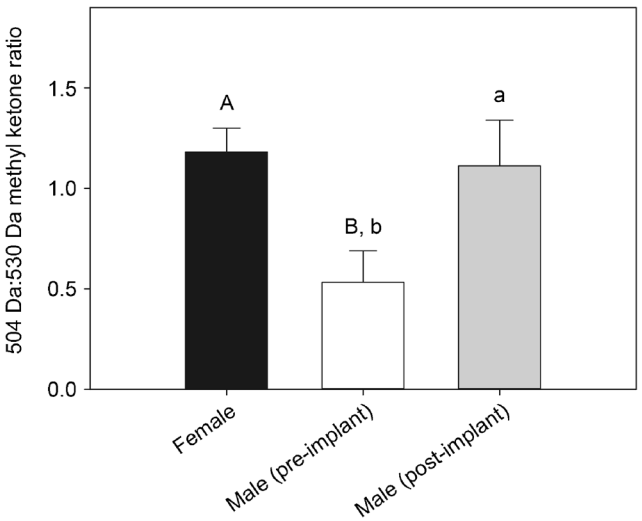

Fig. 3 Female brown treesnakes ($N = 5$) produce a greater amount of pentatriaconten-2-one (504 Da ketomonoene) to heptatriacontadien-2-one (530 Da ketodiene), while the opposite is true for pre-implant control males ($N = 6$). When the same males were implanted with estrogen, the 504:530 ratio was feminized. Bars represent means (+SEM). Upper case letters indicate $P < 0.01$, lower case letters indicate $P < 0.05$)

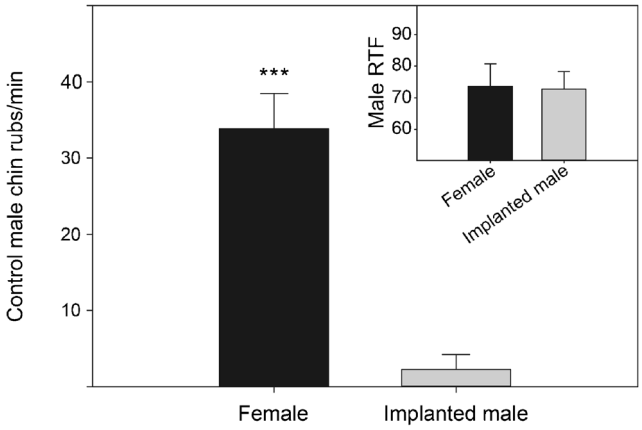

Fig. 4 Male chin rubbing rates were higher (asterisks; $P = 0.002$) when males were paired with females vs. when paired with estrogen-implanted males, but implanted males did elicit weak chin rubbing (an overt reproductive) behavior from males. Bars represent means (+SEM). Inset: unmanipulated male brown treesnakes ($N = 4$) showed the same tongue-flick rate (RTF, tongue-flicks/min) to females as to estrogen-implanted males

did not alter their normal sexual behavior, and no implanted male solicited courtship from males via female-typical behavior.

In the bioassays conducted on Guam with wild males, mean RTFs for males varied across the different extracts presented ($F_{5,59} = 2.90$, $P = 0.023$; Fig. 5). Male RTF was highest for the whole lipid extracts of female shed skin (91.61 ± 7.51 TFs/min) compared to that of male lipid extracts (54.71 ± 10.20 TFs/min)($q_9 = 4.77$, $P = 0.018$). Implanted male F3 + 4 (methyl ketone fractions) and female F3 + 4 isolates elicited marginally higher RTFs from males (85.63 ± 27.00 and 81.43 ± 26.44 TFs/min, respectively) compared to male whole lipid extracts ($q_9 = 3.99$, $P = 0.052$; $q_9 = 3.45$, $P = 0.083$, respectively). All other comparisons were not significant ($P > 0.10$).

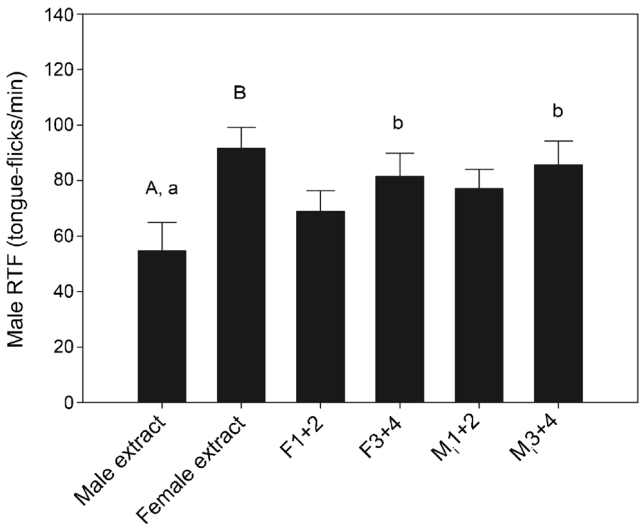

Fig. 5 Wild male brown treesnakes ($N = 10$) on Guam showed variation in chemosensory responses to isolated brown treesnake lipids presented in behavioral arenas. Males discriminated between whole female and whole male lipid extracts from shed skins ($P < 0.001$), and there were marginal increases ($P < 0.10$) in male responses to methyl ketone-containing lipid fractions from females (F3 + 4) and estrogen-implanted males ($M_i3 + 4$) compared to whole male lipid shed skin extracts. The lower polarity lipid fractions (F1 + 2; $M_i1 + 2$) did not elicit stronger responses than the whole male lipid extract. Bars represent means (+SEM). Upper case letters show differences ($P < 0.05$); lower case letters show marginal differences ($0.05 < P < 0.10$)

Discussion

A surprising finding of our study was that male brown treesnakes express the same methyl ketones as females. This has only been documented in one other snake species, the red-sided garter snake, which has a small proportion of males that are female mimics (Mason and Crews 1985). Brown treesnakes produce methyl ketones with very subtle sexual dimorphism, contrary to multiple garter snake species, *Thamnophis* spp., in which females express abundant methyl ketones that are minimally expressed or absent in most males (Mason et al. 1987, 1989; Uhrig et al. 2014). In brown treesnakes, the proportion of pentatriaconten-2-one (504 Da) was greater in females than males, while heptatriacontadien-2-one (530 Da) showed a male-biased expression pattern (Fig. 2). Therefore, pentatriaconten-2-one may be the female-specific signal in this species, a supposition reinforced by the same ketomonoene dominating methyl ketone mixtures of large, female garter snakes (LeMaster and Mason 2002; Parker and Mason 2009, 2012; Uhrig et al. 2014). We also propose that the male-biased ketodiene (heptatriacontadien-2-one) may function as a male-specific chemical signal in *Boiga irregularis*. Following estrogen implantation, male methyl ketone composition shifted to become more typical of females, with post-implant males producing relatively greater quantities of pentatriaconten-2-one and hexatriaconten-2-one, but less heptatriacontadien-2-one, than they did prior to implant.

The ratio of ketomonoenes to ketodienes may be a sex indicator to male brown treesnakes, especially since only one ketomonoene and one ketodiene showed sex-specific patterns of expression. Ratios of methyl ketones affect the attractiveness of pheromone blends in garter snakes and directly control male sexual behavior (LeMaster and Mason 2002), with females becoming more attractive as the ratio of ketomonoenes ("unsaturated") to saturated methyl ketones increases. This same ratio of unsaturated to saturated ketones is augmented by estrogen implantation in male garter snakes and makes them very attractive to wild males (Parker and Mason 2012). The findings we presented in our current paper recapitulate previous results but in a distantly related snake species, *Thamnophis sirtalis*, and it may be that estrogen-activated expression of female skin lipid blends is a conserved physiological process in snakes.

Another interesting finding from our experiments is that female brown treesnakes investigated and responded to male chemical cues during male-female courtship trials. Our observations were qualitative and anecdotal, but male signals can be potent drivers of female behavior in sexual selection (Andersson 1994). Further, many studies in reptiles, especially lizards, have demonstrated a significant role of male pheromone components in attracting females and enabling female mate choice (reviewed in Mason and Parker 2010). Pheromone components from male brown treesnakes could be effective for luring females to traps, and future experiments should be designed to test this idea.

From a behavioral perspective, male brown treesnakes apparently cannot discriminate between skin lipids from females and estrogen-implanted males, suggesting estrogen activates intact, but quiescent, feminization mechanisms in male snakes (Parker and Mason 2012). In our behavioral trials with live focal animals, chemical signals were not the only ones surveyed by males. Indeed, the male-female interaction in brown treesnakes is a mutual courtship process in which the female provides specific feedback to males to accelerate, if not elicit, male sexual behavior. Such behaviors may be proceptive on the part of the female, though the only quantified aspect of female brown treesnake modulation of male behavior is the production of noxious, courtship-inhibiting compounds in cloacal secretions of unreceptive females (Greene and Mason 2003).

If pheromone components have any utility in field-based technologies for improving trapping efforts of invasive brown treesnakes, our behavioral results suggest that whole female lipid extracts would be the most potent mixture for attracting males. Fractionated methyl ketones in our study, as in Greene and Mason (1998), did not increase or even recapitulate the behavioral effects of whole lipid extracts. Instead, we saw a dilution of chemical sampling rate (RTFs), which has been shown in garter snakes when only the methyl ketone blend or individual ketones were presented to males in the field (Mason et al. 1989, 1990). Further, the lipids of brown treesnakes are nonvolatile, which reduces their usefulness as a static, trap-contained component. Trapping is the principle method for removal of brown treesnakes on Guam and is employed preventatively on neighboring islands (Engeman and Vice 2001), but the baits for traps are almost exclusively prey-based. Instead of baiting traps with female lipids, it may be more effective to use whole female lipid extracts in the field to direct males toward traps with chemical trails. Such an application has potential given that, in laboratory trials, male *Boiga* will follow both female and male scent trails (Greene et al. 2001). Manipulative chemoecological approaches that use pheromone components should be compatible with current tools and could be developed to enhance the management of this invasive species.

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References

Albajes R, Konstantopoulou M, Etchepare O, Eizaguirre M, Frérot B, Sans A, Krokos F, Améline A, Mazomenos B (2002) Mating disruption of the corn borer *Sesamia nonagrioides* (Lepidoptera: Noctuidae) using sprayable formulations of pheromone. Crop Prot 21:217–225. https://doi.org/10.1016/S0261-2194(01)00088-6

Andersson MB (1994) Sexual selection. Princeton University Press, Princeton

Bohnenblust EW, Breining JA, Shaffer JA, Fleischer SJ, Roth GW, Tooker JF (2014) Current European corn borer, *Ostrinia nubilalis*, injury levels in the northeastern United States and the value of Bt field corn. Pest Manag Sci 70:1711–1719. https://doi.org/10.1002/ps.3712

Clarke GS, Crossland MR, Shine R (2016) Can we control the invasive cane toad using chemicals that have evolved under intraspecific competition? Ecol Appl 26:463–474. https://doi.org/10.1890/14-2365

El-Sayed AM, Suckling DM, Wearing CH, Byers JA (2006) Potential of mass trapping for long-term pest management and eradication of invasive species. J Econ Entomol 99:1550–1564. https://doi.org/10.1603/0022-0493-99.5.1550

Engeman RM, Vice DS (2001) Objectives and integrated approaches for the control of brown tree snakes. Integr Pest Manag Rev 6:59–76. https://doi.org/10.1023/A:1020441405093

Gaston LK, Shorey HH, Saario CA (1967) Insect population control by the use of sex pheromones to inhibit orientation between the sexes. Nature 213:1155. https://doi.org/10.1038/2131155a0

Greene MJ, Mason RT (1998) Chemically mediated sexual behavior of the brown tree snake, *Boiga irregularis*. Ecoscience 5:405–409. https://doi.org/10.1080/11956860.1998.11682478

Greene MJ, Mason RT (2000) Courtship, mating, and male combat of the brown tree snake, *Boiga irregularis*. Herpetologica 56:166–175

Greene MJ, Mason RT (2003) Pheromonal inhibition of male courtship behaviour in the brown tree snake, *Boiga irregularis*: a mechanism for the rejection of potential mates. Anim Behav 65:905–910. https://doi.org/10.1006/anbe.2003.2137

Greene MJ, Stark SL, Mason RT (2001) Pheromone trailing behavior of the brown tree snake, *Boiga irregularis*. J Chem Ecol 27: 2193–2201. https://doi.org/10.1023/A:1012222719126

Hwang S-Y, Lindroth RL (1997) Clonal variation in foliar chemistry of aspen: effects on gypsy moths and forest tent caterpillars. Oecologia 111:99–108. https://doi.org/10.1007/s004420050213

Johnson NS, Tix JA, Hlina BL, Wagner CM, Siefkes MJ, Wang H, Li W (2015) A sea lamprey (*Petromyzon marinus*) sex pheromone mixture increases trap catch relative to a single synthesized component in specific environments. J Chem Ecol 41:311–321. https://doi.org/10.1007/s10886-015-0561-2

Kimball BA, Stelting SA, McAuliffe TW, Stahl RS, Garcia RA, Pitt WC (2016) Development of artificial bait for brown treesnake suppression. Biol Invasions 18:359–369. https://doi.org/10.1007/s10530-015-1031-z

LeMaster MP, Mason RT (2002) Variation in a female sexual attractiveness pheromone controls male mate choice in garter snakes. J Chem Ecol 28:1269–1285. https://doi.org/10.1023/A:1016294003641

Mason RT (1992) Reptilian pheromones. In: Gans C, Crews D (eds) Biology of the Reptilia, vol 18. University of Chicago Press, Chicago, pp 114–228

Mason RT, Crews D (1985) Female mimicry in garter snakes. Nature 316: 59–60. https://doi.org/10.1038/316059a0

Mason RT, Parker MR (2010) Social behavior and pheromonal communication in reptiles. J Comp Physiol A 196:729–749. https://doi.org/10.1007/s00359-010-0551-3

Mason RT, Chinn JW, Crews D (1987) Sex and seasonal differences in the skin lipids of garter snakes. Comp Biochem Physiol B 87:999–1003. https://doi.org/10.1016/0305-0491(87)90424-X

Mason RT, Fales HM, Jones TH, Pannell LK, Chinn JW, Crews D (1989) Sex pheromones in snakes. Science 245:290–293. https://doi.org/10.1126/science.2749261

Mason RT, Jones TH, Fales HM, Pannell LK, Crews D (1990) Characterization, synthesis, and behavioral responses to sex attractiveness pheromones of red-sided garter snakes (*Thamnophis sirtalis parietalis*). J Chem Ecol 16:2353–2369. https://doi.org/10.1007/BF01026943

Mathies T, Miller LA (2003) Cool temperatures elicit reproduction in a biologically invasive predator, the brown treesnake (*Boiga irregularis*). Zoo Biol 22:227–238. https://doi.org/10.1002/zoo.10084

Mathies T, Levine B, Engeman R, Savidge JA (2013) Pheromonal control of the invasive brown treesnake: potency of female sexual attractiveness pheromone varies with ovarian state. Int J Pest Manage 59: 141–149. https://doi.org/10.1080/09670874.2013.784374

McNeil JN (1991) Behavioral ecology of pheromone-mediated communication in moths and its importance in the use of pheromone traps. Annu Rev Entomol 36:407–430. https://doi.org/10.1146/annurev.en.36.010191.002203

Mortensen HS, Dupont YL, Olesen JM (2008) A snake in paradise: disturbance of plant reproduction following extirpation of bird flower-visitors on Guam. Biol Conserv 141:2146–2154. https://doi.org/10.1016/j.biocon.2008.06.014

Murata Y, Yeh HJ, Pannell LK, Jones TH, Fales HM, Mason RT (1991) New ketodienes from the integumental lipids of the Guam brown tree snake, *Boiga irregularis*. J Nat Prod 54: 233–240. https://doi.org/10.1021/np50073a024

Musso AE, Gries R, Zhai H, Takács S, Gries G (2017) Effect of male house mouse pheromone components on behavioral responses of mice in laboratory and field experiments. J Chem Ecol 43: 215–224. https://doi.org/10.1007/s10886-017-0819-y

Parker MR, Mason RT (2009) Low temperature dormancy affects the quantity and quality of the female sexual attractiveness pheromone in red-sided garter snakes. J Chem Ecol 35:1234–1241. https://doi.org/10.1007/s10886-009-9699-0

Parker MR, Mason RT (2011) Pheromones in snakes: history, patterns and future research directions. In: Aldridge RD, Sever DM (eds) Reproductive biology and phylogeny of snakes. CRC Press, Boca Raton, pp 551–572

Parker MR, Mason RT (2012) How to make a sexy snake: estrogen activation of female sex pheromone in male red-sided garter snakes. J Exp Biol 215:723–730. https://doi.org/10.1242/jeb.064923

Parker MR, Mason RT (2014) A novel mechanism regulating a sexual signal: the testosterone-based inhibition of female sex pheromone expression in garter snakes. Horm Behav 66:509–516. https://doi.org/10.1016/j.yhbeh.2014.07.007

Reardon RC, Leonard D, Mastro V, Leonhardt B, Mclane W, Talley S, Thorpe K, Webb R (1998) Using mating disruption to manage gypsy moth: a review. USDA Forest Service, Washington, D.C.

Rodda GH, Fritts TH (1992) The impact of the introduction of the colubrid snake Boiga irregularis on Guam's lizards. J Herpetol 26:166–174. https://doi.org/10.2307/1564858

Rogers HS, Buhle ER, HilleRisLambers J, Fricke EC, Miller RH, Tewksbury JJ (2017) Effects of an invasive predator cascade to plants via mutualism disruption. Nat Commun 8:14557. https://doi.org/10.1038/ncomms14557

Saunders G, Cooke B, McColl K, Shine R, Peacock T (2010) Modern approaches for the biological control of vertebrate pests: an Australian perspective. Biol Control 52:288–295. https://doi.org/10.1016/j.biocontrol.2009.06.014

Savidge JA (1987) Extinction of an island forest avifauna by an introduced snake. Ecology 68:660–668. https://doi.org/10.2307/1938471

Smith JB, Turner KL, Beasley JC, DeVault TL, Pitt WC, Rhodes OE (2016) Brown tree snake (*Boiga irregularis*) population density and carcass locations following exposure to acetaminophen. Ecotoxicology 25:1556–1562. https://doi.org/10.1007/s10646-016-1711-1

Takács S, Gries R, Zhai H, Gries G (2016) The sex attractant pheromone of male brown rats: identification and field experiment. Angew Chem Int Ed 55:6062–6066. https://doi.org/10.1002/anie.201511864

Takács S, Gries R, Gries G (2017) Sex hormones function as sex attractant pheromones in house mice and brown rats. Chembiochem 18: 1391–1395. https://doi.org/10.1002/cbic.201700224

Traveset A, Richardson DM (2006) Biological invasions as disruptors of plant reproductive mutualisms. Trends Ecol Evol 21:208–216. https://doi.org/10.1016/j.tree.2006.01.006

Uhrig EJ, LeMaster MP, Mason RT (2014) Species specificity of methyl ketone profiles in the skin lipids of female garter snakes, genus *Thamnophis*. Biochem Syst Ecol 53:51–58. https://doi.org/10.1016/j.bse.2013.12.016

Wiles GJ, Bart J, Beck RE, Aguon CF (2003) Impacts of the brown tree snake: patterns of decline and species persistence in Guam's avifauna. Conserv Biol 17:1350–1360. https://doi.org/10.1046/j.1523-1739.2003.01526.x